Chemical Dosimetry in Somatic Cells and Its Utility to Mutagenesis

by James B. Patterson*

In assessing the potential health hazard a given mutagen might present to man, a number of extrapolations must be made from the test systems which are currently available. For the L5178Y mouse lymphoma cell assay system that we use, these extrapolations include: somatic versus germinal cells; single cells versus whole animals; transformed versus "normal" cells; and mouse versus human cells. All of these factors represent potentials for ameliorating or enhancing the mutagenic effects of a given agent, especially by altering the extent of interaction between mutagen and DNA. By quantitating this interaction for the various test systems, the confidence with which mutagenic data can be meaningfully extrapolated to man is greatly improved.

Problems of dosage interpretation of mutagenic agents arise as a consequence of innate differences in the many different mutagen assay systems. Dr. Rall has outlined some of these problems earlier (*1*). It is important for us to realize that the interactions of a chemical agent with DNA in a test tube may not mimic the reaction that occurs in a mammalian cell. While the interaction itself may be interesting and educational, it may or may not relate to the actual mutagenic initiation event in an intact animal. Certain classes of environmental agents are known to interact either physically or chemically with DNA. Any foreign chemical which has an affinity for and a tendency to react with specific components of genetic material may possess the potential for causing a mutagenic event to occur.

Alkylating agents make up the largest group of chemicals which are known to act as mutagens. Included in this group are nitrogen, sulfur, and oxygen mustards; nitrosamines and nitrosamides; epoxides, lactones, aziridines, aldehydes, alkane sulfonic esters, dialkyl sulfates, and many related derivatives. Other chemicals which interact with DNA are a variety of antibiotics and mycotoxins, nitrous acid and nitrites, bisulfites, peroxides, acridines, heavy metal salts, and polynuclear aromatic hydrocarbons. In addition, some chemical compounds are metablically converted to new compounds which can then react with DNA.

The half-life of a reactive compound further governs whether an agent can enter the nucleus in sufficient quantities to interact with the genetic apparatus. For instance, if the compound in question has a halflife of less than 1 min, the chance of the compound getting into a germ cell nucleus before reacting with extracellular or cytoplasmic nucleophils is relatively small.

Because of the variety of reaction options presented to a chemical agent entering a cell, it becomes extremely difficult to predict how an untested agent will react in any given mutagenic assay system. Since no single assay system now available can detect the whole spectrum of mutagenic effects, it

*Mutagenesis Branch, National Institute of Enviromental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709.

is important to study the interaction of an agent with cells and cell components in several assay systems. Increasing credence is being placed on mutation studies in somatic mammalian cells. This makes it necessary to compare and contrast the binding of chemical agents to cellular components in germ cells and somatic cells from the same species. The theoretical and practical attractiveness of some mammmalian somatic cell culture assay systems has already been discussed in these proceedings.

The relationship between the number of mutagenic events induced and the number of insults visited upon specific cellular macromolecules by chemical compounds is a central one in the study of chemical mutagenesis. This relationship can be referred to as a dosimetric one. By performing well-designed dosimetry experiments, an investigator can determine how much of an administered chemical is actually reacting with DNA, RNA, or proteins (e.g., general proteins or specific ones such as histones or DNA polymerase). This short discussion will be restricted to the interactions of alkylating agents with DNA, although one should appreciate that alterations induced into the enzymes of DNA metabolism and replication can significantly alter the fidelity of replication. It has been reported recently (*2*) that mutations introduced into the gene for phage T4 DNA polymerase can cause the enzyme to act as a mutator (decreased fidelity), an antimutator (increased fidelity), or the mutation rate may be unaltered. While these observations concern mutations in the enzyme instead of nongenetic lesions (e.g., alkylation of an amino acid residue in or near an active-site), it is theoretically possible to understand how an alkylation of DNA polymerase in mammalian cells could result in an increased rate of mutation.

Nearly all of the dosimetric studies performed until now have been done in the name of chemical carcinogenesis; however, most of the experimental conditions apply themselves as well to chemical mutagenesis. Such studies require that the DNA be isolated free of all other cellular components, then degraded and chromatographed. These operations become necessary in order to ascertain to what extent certain chemicals have a tendency to react with specific components of DNA.

DNA has generally been degraded by some form of acid hydrolysis. However, it has been appreciated for at least a decade that some alkylated bases are dealkylated by the conditions necessary for hydrolysis to occur [deoxy-(0–6)-methylguanosine yields guanine on hydrolysis with perchloric acid] (*3*). In the presence of alkali, 1-methyladenosine is converted to *N*–6-methyladenosine (*4*); 2-amino-6-methoxypurine is dealkylated at pH 1, 100° C (*5*). These are simply examples to illustrate that no single-step chemical method of degrading DNA is likely to yield all of the products of alkylation.

An encouraging development in the hydrolysis of DNA has been the introduction of controlled enzymatic hydrolysis methods. Treatment of DNA with DNase yields an oligonucleotide mixture; addition of snake venom phosphodiesterase or lambda exonuclease (6) yields 5′-mononucleotides, further incubation with bacterial alkaline phosphatase yields nucleosides from the mononucleoside-5′-phosphate mixture (*7*). A complicating factor in the use of enzymatic hydrolysis is the evidence that some chemically altered DNA may be partially resistant to enzyme degradation (*8*).

The R_f values and spectral properties of a large number of DNA hydrolysis products and base derivatives have been determined for various solvent systems (*7,9*). The basic methods now available for the separation of nucleotides are paper chromatography (*9–11*) and column chromatography (*9,12*) [e.g., Dowex-1 (*13,14*), DEAE–cellulose, 7*M* urea (*15,16*), DEAE–Sephadex, 7M urea (*17*)]. Nucleosides can be separated by chromatography on columns of Dowex-50 (ammonium form) (*18–20*), kieselguhr (aqueous and organic solvents) (*7*), Sephadex LH–20 (organic solvents) (*21*), and Sephadex G–10 (water or buffers) (*18*). Sephadex G–10 (*22,23*) and Dowex-50 (H^+-form eluted with 2*N* HCl) (*18,24*) are the

methods of choice for separating bases. None of these chromatographic techniques will provide absolute separation of all the altered bases that might occur in alkylated DNA. However, a prudent combination of the more effective methods should allow detection of most of the altered bases as they exist in the cell.

The chromatographed products are usually detected and identified by their ultraviolet absorption properties in the 210–350 nm range. Radioactively labeled mutagens provide greater sensitivity for the detection and identification of small quantities of DNA products. Of course, this technique requires that the labeled moiety of the mutagen becomes chemically bound to a component of the NA. Sugimura et al. (*25*) used doubly-labeled *N*-^{3}H-methyl-*N'*-nitro-*N*-nitroso-^{14}C-guanidine (MNNG) in *in vitro* binding experiments to macromolecules. The ^{3}H-methyl was preferably bound to DNA and poly A (low for RNA and poly U) and the ^{14}C-guanidine was preferably bound to proteins (histone > cytochrome C > RNase > globulin). Lijinsky et al. (*26*) employed mass spectroscopy to determine that dimethylnitrosamine (labeled with deuterium in the methyl group) injected into rats produced 7-methylguanine in liver DNA in which the methyl group was an intact CD_3. This provided proof that diazomethane is not the methylating species derived from dimethylnitrosamine as had been suggested.

Investigators have used combinations of these various methods in an effort to produce, isolate, and identify those altered bases which may be directly responsible for the initiation of a carcinogenic event. The answers can be applied as well to questions related to anomalous base pairing leading to a mutagenic event.

The major products of DNA alkylation are generally 7-alkylguanine, 3-alkyladenine and 7-alkyladenine; a number of other minor alkylated base products occur, the respective levels varying with the alkylating agent and the exposure conditions.

Lawley and Shah (*18*) examined the alkylation products of methyl nitrosourea (MNU) and dimethyl sulfate (DMS) in RNA *in vitro*. They found that 7-methylguanine is the major product of reaction with both compounds but the distribution of minor methylation products differs significantly. The principle minor product of DNA alkylation by MNU and MNNG is O–6-methylguanine; however, it is not detected upon alkylation with DMS. The principal minor products from methyl methanesulfonate (MMS) and DMS alkylation are 1-methyladenine and 3-methylcytosine (*27*). Even though the O–6 atom of guanine is involved in hydrogen bonding, the relative degree of O–6 alkylation in DNA is about twice that in RNA. Loveless (*28*) reacted MNU with deoxyguanosine and determined that the O–6 methyl derivative accounted for about 10% of the total alkylation products; the ethyl nitrosourea (ENU) reaction product yielded three to four times as much O–6-ethylguanine. When MMS, ethyl methanesulfonate (EMS), and DMS were reacted with deoxyguanylic acid, only EMS yielded the O–6 alkyl product. Loveless (*29*) had earlier determined that whereas both MMS and EMS produce 7-alkylguanine derivatives in the DNA of T-even phages, only EMS is mutagenic. However, both MNU and ENU were found to be mutagenic in T_2 phage (*30*). These data led Loveless to hypothesize that since the 1-position of guanine no longer bears a proton after O–6-alkylation, such a reaction fosters anomalous base pairing.

Several investigators have since found O–6-alkylguanine after treating cells with alkyl-nitrosamides, alkyl-nitrosamidines and EMS. Lawley and Thatcher (*5*) found the *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) treatment to yield about 7% O–6-methyl guanine both *in vitro* and in mouse cells, whereas DMS did not yield that product. MNNG is known to induce mutations in *Neurospora*, 81% of which exhibit nonpolarized complementation patterns, indicating the probable presence of a complete polypeptide chain; among MMS-induced mutants the figure is only about 31% (*31*). MMS also induced chromosome abnormalities, whereas MNNG did not. These data show that MNNG

acts as a specific inducer of point mutations, indicating that MNNG and other alkylnitrosamides and alkylnitrosamidines, as well as EMS, may have different reactive specificities than MMS and DMS.

The S_N1 chemical reaction mechanism involves the production of a reactive carbonium ion, whereas the S_N2 reaction mechanism involves the formation of a transition complex between the nucleophilic and the electrophilic species. S_N1 type reagents attack chemical groups independently of their nucleophilicity; S_N2 reagents are increasingly reactive toward more nucleophilic groups. The sites in DNA generally attacked by MMS and DMS are groups of known high nucleophilicity. Since the S_N1 reaction is more indiscriminate in its reactive target, it may well be that alkylation of the O–6 of guanine is indicative of an agent which reacts by the S_N1 mechanism (*5*). The alkylation of the O–6 of guanine in DNA could therefore be related to the high mutagenic efficiency of S_N1 type alkylating agents. The presence of 3-methylguanine residues in DNA treated with MMS and DMS provides a possible answer for the mutagenicity of S_N2 alkylating agents, since 3-methylguanine is a potentially mispairing base (*32*).

REFERENCES

1. Rall, D. P. Pharmokinetic and other factors related to mutagenicity testing. Environ. Health Perspect. **No. 6**: 67 (1973).
2. Muzyczka, N., Poland, R. L., and Bessman, M. J. Studies on the biochemical basis of spontaneous mutation. I. A comparison of the deoxyribonucleic acid polymerases of mutator, antimutator, and wild type strains of bacteriophage T4. J. Biol. Chem. **247**: 7116 (1972).
3. Friedman, O. M., Mahapatra, G. N., and Stevenson, R. The methylation of deoxyribonucleosides by diazomethane. Biochim. Biophys. Acta **68**: *144 (1963)*.
4. Brookes, R., and Lawley, P. D. The methylation of adenosine and adenylic acid. J. Chem. Soc. 539 (1960).
5. Lawley, P. D., and Thatcher, C. J. Methylation of deoxyribonucleic acid in cultured mammalian cells by *N*-methyl-*N'*-nitro-*N*-nitrosoguandine. The influence of cellular thiol concentrations on the extent of methylation and the 6-oxygen atom of guanine as a site of methylation. Biochem, J. **116**: 693 (1970).
6. Little, J. W. An exonuclease induced by bacteriophage lambda. In: Methods in Enzymology, Vol. 12A: L. Grossman and K. Moldave, Eds. Academic Press, New York–London, 1967 p. 263.
7. Hall, R. H. Partition chromatography of nucleic acid components (isolation of minor nucleosides). In: Methods in Enzymology, Vol. 12A. L. Grossman and K. Moldave, Eds. Academic Press, New York–London, 1967, p. 305.
8. Kriek, E. Difference in binding of 2-acetylaminofluorene to rat liver deoxyribonucleic acid and ribosomal ribonucleic acid *in vivo*. Biochim. Biophys. Acta **161**: 273 (1968).
9. Brookes, P., and Lawley, P. D. Effects on DNA: chemical methods. In: Chemical Mutagens: *Principles and Methods for their Detection*. Vol. 1, A. Hollaender, Ed. Plenum Press, New York–London, 1971, p. 121.
10. Randerath, K., and Randerath, E. Thin-layer separation methods for nucleic acid derivatives. In: Methods in Enzymology, Vol. 12A. L. Grossman and K. Moldave, Eds., Academic Press, New York–London, 1967, p. 323.
11. Laskowski, M. Paper chromatography and characterization of oligodeoxyribonucleotides. In: Methods in Enzymology, Vol. 12A. L. Grossman and K. Moldave, Eds. Academic Press, New York–London, 1967, p. 281.
12. Tener, G. M. Ion exchange chromatography in the presence of urea. In: Methods in Enzymology, Vol. 12A. L. Grossman, and K. Moldave, Eds. Academic Press, New York–London, 1967, p. 398.
13. Solymosy, F., Tener, G. M., and Reichmann, M. E. Partial nucleotide sequences in turnip yellow mosaic virus RNA. Virology **27**: 409 (1965).
14. Miura, K.-I., and Hayashi, Y. Dowex 1 chromatography of oligonucleotides. In: Methods in Enzymology, Vol. 12A. L. Grossman and K. Moldave, Eds. Academic Press, New York–London. 1967, p. 390.
15. Tomlinson, R. V., and Tener, G. M. The effect of urea, formamide, and glycols on the secondary binding forces in the ion-exchange chromatography of polynucleotides in DEAE–cellulose. Biochemistry **2**: 697 (1963).
16. Bartos, E. M., Rushinzky, G. W., and Sober, H. A. Chromatography of ribonuclease T1 digests of RNA on the DNA-cellulose in 7*M* urea. Biochemistry **2**: 1179 (1963).
17. Rushinzky, G. W., Bartos, E. M., and Sober, H. A. Chromatography of mixed oligonucleotides on DEAE–Sephadex. Biochemistry **3**: 626 (1964).
18. Lawley, P. D., and Shah, S. A. Methylation of ribonucleic acid by the carcinogens dimethyl sulfate, *N*-methyl-*N*-nitrosourea and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Comparisons of

chemical analyses at the nucleoside and base levels. Biochem. J. 128: 117 (1972).

19. Junowicz, E., and Spencer, J. H. Rapid separation of nucleosides and nucleotides by cation-exchange column chromatography. J. Chromatog. 44: 342 (1969).
20. Uziel, M., Koh, C. K., and Cohn, W. E. Rapid ion-exchange chromatographic microanalysis of ultraviolet-absorbing materials and its application to nucleosides. Anal. Biochem. 25: 77 (1968).
21. Kriek, E. On the mechanism of action of carcinogenic aromatic amines. I. Binding of 2-acetylaminofluorene and *N*-hydroxy-2-acetylaminofluorene to rat liver nucleic acids *in vivo*. Chem. Biol. Interactions 1: 3 (1969).
22. Lawley, P. D. Methylation of DNA by *N*-methyl-*N*-nitrosourethane and *N*-methyl-*N*-nitroso-*N'* nitroguanidine. Nature 218: 580 (1968).
23. Braun, R. Separation of bases, nucleosides and nucleotides on Sephadex G–10. Biochim. Biophys. Acta 142: 267 (1967).
24. Cohn, W. E. The separation of purine and pyrimidine bases and of nucleotides by ion exchange. Science 109: 377 (1949).
25. Sugimura, T., et al. Reaction of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine with protein. Biochim. Biophys. Acta 170: 427 (1968).
26. Lijinsky, W., Loo, J. and Ross, A. E. Mechanism of alkylation of nucleic acids by nitroso-dimethylamine. Nature 218: 1174 (1968).
27. Tropp, B. E., Law, J. H., and Hayes, J. M. Studies on the mechanism of biological methylation of nucleic acids. Biochemistry 3: 1837 (1964).
28. Loveless, A. Possible relevance of O–6 alkylation of deoxyguanosine to the mutagenicity and carcinogenicity of nitrosamines and nitrosamides. Nature 223: 206 (1969).
29. Loveless, A. The influence of radiomimetic substances on deoxyribonucleic acid synthesis and function studied in *Escherichia coli*- phage systems. III. Mutation of T2 bacteriophage as a consequence of alkylation *in vitro* the uniqueness of ethylation. Proc. Roy. Soc. (London) B150: 497 (1959).
30. Loveless, A., and Hampton, C. Inactivation and mutation of coliphage T2 by N-methyl and N-ethyl-N-nitrosourea. Mutation Res. 7: 1 (1968).
31. Malling, H. V., and de Serres, F. J. Mutagenicity of alkylating carcinogens. Ann. N. Y. Acad. Sci. 163: 788 (1969).
32. Lawley, P. D., Orr, D. J., and Shah, S. A. Reaction of alkylating mutagens and carcinogens with nucleic acids: *N*-3 of guanine as a site of alkylating by N-methyl-N-nitrosourea and dimethyl sulfate, Chem. Biol. Interactions 4: 431 (1971/2).